NATO AC/225 LG/7-SIBCA Second International Training Exercise on the Identification of Mid-Spectrum Agents

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NATO AC/225 LG/7-SIBCA SECOND INTERNATIONAL TRAINING EXERCISE ON THE IDENTIFICATION OF MID-SPECTRUM AGENTS

by

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EXECUTIVE SUMMARY

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Introduction: The Canadian Forces (CF) may be called on to perform peacekeeping or peacemaking operations in regions of the world where there is a significant threat of chemical/biological warfare agent use. To operate effectively in these theatres the CF must be able to identify the exact nature of the chemical/biological agent(s). Mass spectrometry (MS) is a powerful analytical technique for the identification of both known and unknown compounds and DRE Suffield, in conjunction with its NATO allies, is currently investigating this instrumental technique in fulfilment of CF and NATO agent detection and identification requirements.

Results: In 1999, the SIBCA subgroup conducted the second international training exercise on the identification of mid-spectrum agents. The objective of this exercise, hosted by Canada, was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents. The compounds were selected from the SIBCA list of mid-spectrum agents and distributed as unknowns to the laboratories. Seven national laboratories, one each from Canada, France, Germany, Italy, Netherlands, Norway and the United States participated in the exercise. Canada, as the host country, analyzed the samples prior to the exercise as part of the sample selection process. The samples were distributed to the laboratories by 1 February 1999 with the constraint that the analysis be completed by the end of the month. Within that timeframe, a limit of five working days was placed upon the analysis of the samples

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. All the participating laboratories were able to provisionally identify the first sample as α-Conotoxin MI and the second sample as apamin. The Netherlands and Germany through further experiments were able to confirm the identification of α-Conotoxin MI and apamin. In addition to the information required for confirmed identification, the Netherlands used nuclear magnetic resonance spectrometry and an authentic reference sample to unambiguously identify apamin.

Significance of Results: The CF may be deployed in regions of the world where there is a significant threat of chemical/biological warfare agent use. Identification of the agent is of importance since the results of such analyses would contribute to the development of strategic and political positions regarding future Canadian military operations and would facilitate the dissemination of technical advice to in-theater field commanders and medical personnel.

Future Goals: The CB threat spectrum includes chemical and biological warfare agents and toxins of biological origin in the "mid-spectrum" between these agents. The identification research effort has been focused on the detection and identification of these toxins of biological origin. Use of these warfare agents could easily go unconfirmed, as analytical methods have not been fully developed for their identification. DRE Suffield is now actively addressing this deficiency through the application and development of MS methods for the identification of these agents.

ABSTRACT

In February 1999, the NATO LG/7-SIBCA subgroup conducted the second international training exercise on the identification of mid-spectrum agents. The objective of this training exercise, hosted by Canada, was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents. The compounds were selected from the SIBCA list of mid-spectrum agents and distributed as unknowns to the laboratories. Seven national laboratories, one each from Canada, France, Germany, Italy, Netherlands, Norway and the United States, participated in the exercise.

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. All the participating laboratories were able to provisionally identify the first sample as α-Conotoxin MI and the second sample as apamin. The Netherlands and Germany through further experiments were able to confirm the identification of α-Conotoxin MI and apamin. In addition to the information required for confirmed identification, the Netherlands used nuclear magnetic resonance spectrometry and an authentic reference sample to unambiguously identify apamin.

Keywords: NATO
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Mid-spectrum agents
Identification
Training exercise
α-Conotoxin MI
Apamin

TABLE OF CONTENTS

Contributing Laboratories ii
Executive Summary iii
Abstract iv
Table of Contents v
List of Tables v
List of Figures vi
INTRODUCTION 1
RESULTS 2
DISCUSSION 16
CONCLUSIONS 19
ANNEX I 21

LIST OF TABLES

Table I. Monoisotopic and Average Molecular Masses Determined for MSA Sample 001 ... 3

Table II. LC/MS/MS Results Obtained by France for T3 Fragment 8

Table III. Monoisotopic and Average Molecular Masses Determined for MSA Sample 002 .. 10

Table IV. Tryptic Digest Mass Map Obtained by France for MSA Sample 002 13

Table V. Level of Identification Reached by Participating Laboratories 16

Table VI. Intensity of Isotopic Clusters Obtained by Participating Laboratories for MSA Sample 001 18

Table VII. Intensity of Isotopic Clusters Obtained by Participating Laboratories for MSA Sample 002 18

LIST OF FIGURES

Figure 1. ESI-MS spectrum acquired by Italy during analysis of MSA Sample 001 using an orifice voltage of 70V. 3

Figure 2. ESI-MS spectrum acquired by Germany during the analysis of MSA sample 001 following reduction with 1.5 % 2-mercaptoethanol. Molecular mass was increased by 4 Da indicating the presence of 2 disulfide bridges 4

Figure 3. ESI-MS/MS product ion spectrum acquired by the Netherlands for the $[M+2H]^{2+}$ precursor ion from reduced underivatized MSA sample 001 at a sampling cone voltage of 30V. Lower figure is Max Ent transformed spectrum with markings of the sequence and a, b and y series ions 5

Figure 4. ESI-MS spectra obtained by France during the LC-MS analysis of the tryptic digestion of reduced MSA sample 001; a) T2 fragment, b) T3 fragment and c) T2-NH_3 fragment 6

Figure 5. ESI-MS/MS product ion spectrum acquired by France for the $[M+H]^+$ precursor ion (542.5 Da) for tryptic fragment T3 of MSA Sample 001 7

Figure 6. ESI-MS spectrum acquired by Norway during the analysis of MSA Sample 002 .. 10

Figure 7. ESI-MS spectrum acquired by the United States during the analysis of MSA sample 002 following reduction and alkylation (iodoacetamide). Molecular mass was increased by 232 Da indicating the presence of two disulfide bridges 11

Figure 8. LC-ESI-MS; a) total ion current chromatogram and b) reconstructed ion chromatogram acquired by France during analysis of tryptic digestion products from reductively alkylated (iodoacetamide) MSA sample 002 12

Figure 9. LC-ESI-MS spectra acquired by France for tryptic fragments; a) T1, T4, T3+T4, b) T2 and c) T1+T2 during analysis of reductively alkylated (iodoacetamide) MSA sample 002 13

Figure 10. ESI-MS/MS product ion spectrum acquired by the Netherlands for the $[T2+2H]^{2+}$ precursor ion (518.74 Da) from reduced and alkylated (4-vinylpyridine) MSA sample 002 14

INTRODUCTION

NATO may be called upon to deploy military forces in support of peacekeeping or battlefield operations in regions of the world where there is a significant threat of the use of chemical/biological warfare (CBW) agents. To operate effectively in these theatres, NATO forces must be able to detect and identify CBW agents.

In 1996, the NATO AC/225 LG/7 subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted an international training exercise on the identification of peptides and proteins. Seven national laboratories from Canada, Denmark, Netherlands, Norway, United Kingdom and United States (2 laboratories) participated in the international training exercise whose objective was to determine the molecular mass of the unknown peptides and using any available technique, to determine partial or complete amino acid sequences for the unknown peptides.

In 1999, the SIBCA subgroup conducted the second international training exercise on the identification of mid-spectrum agents. The objective of this exercise, hosted by Canada, was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents. The compounds were selected from the SIBCA list of mid-spectrum agents and distributed as unknowns to the laboratories. Seven national laboratories, one each from Canada, France, Germany, Italy, Netherlands, Norway and the United States, participated in the exercise. Canada, as the host country, analyzed the samples prior to the exercise as part of the sample selection process. The samples were distributed to the laboratories by 1 February 1999 with the constraint that the analysis be completed by the end of the month. Within that timeframe, a limit of five working days was placed upon the analysis of the samples.

RESULTS

DRES MSA Sample 001

The first sample labelled "DRES MSA Sample 001" contained 1mg of α-Conotoxin MI (Bachem, Torrance CA, USA). The amino acid sequence and molecular mass of α-Conotoxin MI are given below.

Gly-Arg-Cys-Cys-His-Pro-Ala-Cys-Gly-Lys-Asn-Tyr-Ser-Cys-NH_2

Monoisotopic molecular mass:	1492.55807 Da
Average molecular mass:	1493.73681 Da
CAS number:	83481-45-2
Disulfide Bridges:	Cys 3-8 and Cys 4-14

Molecular Mass Determination

All the participating laboratories determined the molecular mass of the sample using electrospray ionization mass spectrometry. In addition, Germany used both SDS-PAGE and gel permeation chromatography to obtain an initial estimate of the molecular mass of the sample. Figure 1 illustrates the electrospray mass spectrum acquired by Italy during the analysis of MSA sample 001. The mass spectrum contained $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$, and $[M+4H]^{4+}$ ions from which the molecular mass was determined. Table I lists the monoisotopic or average molecular masses obtained by each country. Once the laboratories obtained the molecular masses for the sample, and searched available databases, they were able to provisionally identify the sample as α-Conotoxin MI.

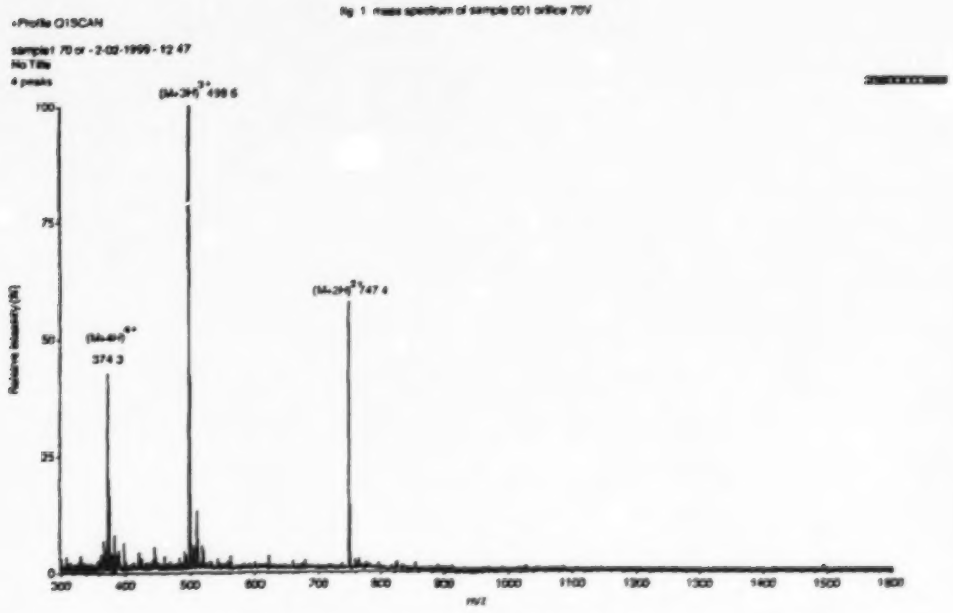

Figure 1. ESI-MS spectrum acquired by Italy during analysis of MSA Sample 001 using an orifice voltage of 70V.

Table I. Monoisotopic and Average Molecular Masses Determined for MSA Sample 001

Country	Instrument	Monoisotopic Molecular Mass 1492.55807 Da	Error ppm	Average Molecular Mass 1493.73681 Da	Error ppm
France	Bruker Ion Trap	1492.45±0.07	72		
Germany	HP 1100 MSD	1492.7	95		
Italy	PE Sciex	1492.8±0.2	162		
Netherlands	Micromass Q-TOF	1492.557±0.010	1		
Norway	Micromass Quattro			1493.19±0.01	366
U.S.	Finnigan TSQ7000			1493.35±0.26	259
Canada[a]	VG AutoSpec	1492.55±0.02	5		

[a] host country

Reductive Alkylation

Once MSA sample 001 had been provisionally identified as α-Conotoxin MI, a number of laboratories supported their identification by checking for the presence of disulfide bonds. Reduction of the disulfide bonds was in some cases followed by alkylation of the cysteine. The resulting change in the molecular mass of the analyte was determined by mass spectrometry. Figure 2 illustrates the ESI mass spectrum acquired by Germany during analysis of MSA sample 001 following reduction with 1.5% 2-mercaptoethanol (MCE). France, Norway, Germany, the Netherlands and the United States were able to confirm the presence of four cysteines and two intramolecular disulfide bridges.

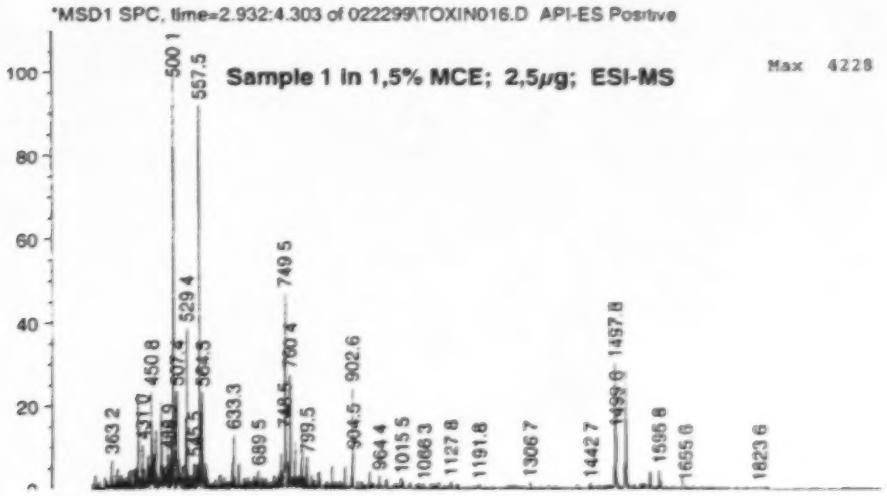

Figure 2. ESI-MS spectrum acquired by Germany during the analysis of MSA sample 001 following reduction with 1.5 % 2-mercaptoethanol. Molecular mass was increased by 4 Da indicating the presence of 2 disulfide bridges.

Sequencing of Intact Conotoxin

α-Conotoxin MI is a relatively small peptide and both France and the Netherlands acquired sequence data for the reduced toxin. Figure 3 illustrates the ESI-MS/MS product ion spectrum acquired by the Netherlands for the $[M+2H]^{2+}$ precursor ion. Both laboratories used the $[M+2H]^{2+}$ precursor ion and obtained almost complete sequence information from the b or y series.

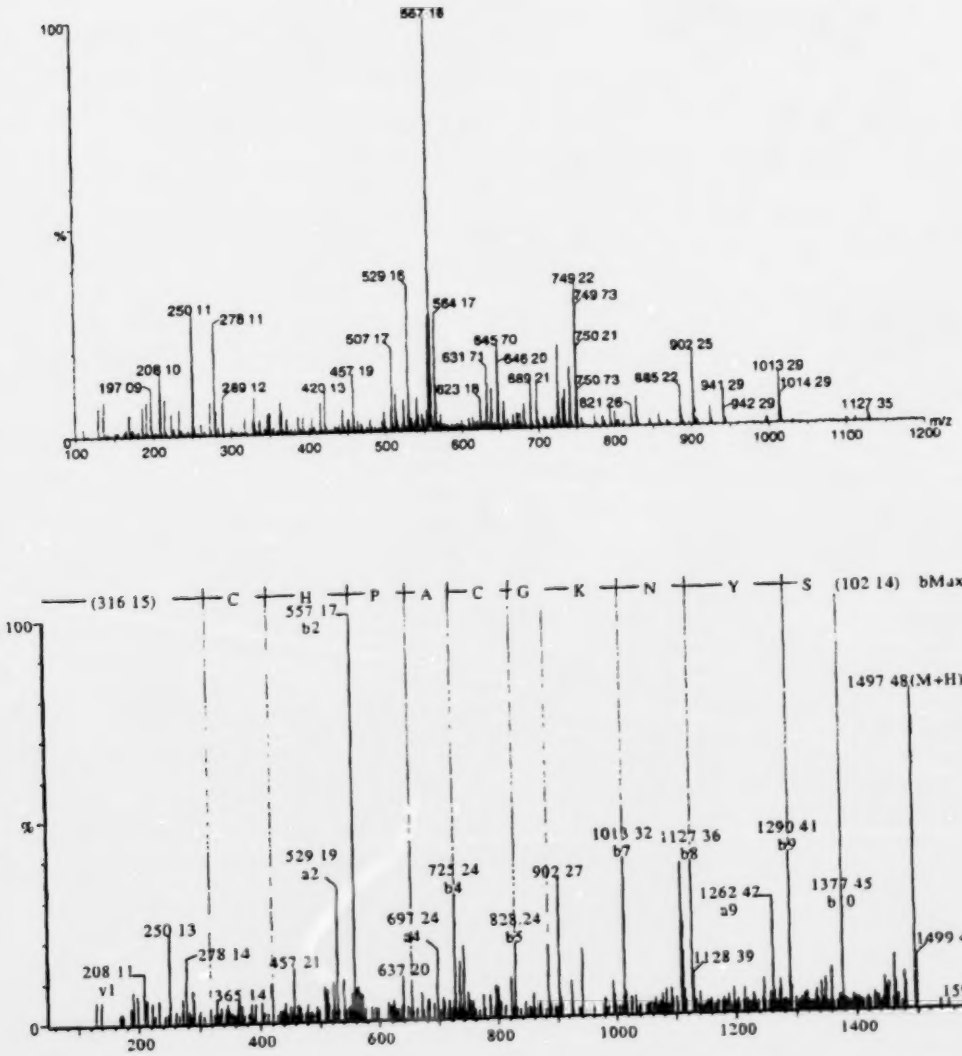

Figure 3. ESI-MS/MS product ion spectrum acquired by the Netherlands for the $[M+2H]^{2+}$ precursor ion from reduced underivatized MSA sample 001 at a sampling cone voltage of 30V. Lower figure is Max Ent transformed spectrum with markings of the sequence and a, b and y series ions.

Enzymatic Cleavage

As the molecular mass of mid-spectrum agent increases, enzymes such as trypsin are typically used to digest the sample. The molecular masses and sequences of the lower mass fragments resulting from the digestion can then be determined. France and the Netherlands both carried out tryptic digestions and analyzed the digestion products. Figure 4 illustrates the mass spectra, acquired by France, during the LC-MS analysis of the tryptic digestion of reduced MSA sample 001. Two of the three possible tryptic fragments (T2 and T3) were detected. A third compound, the result of the loss of NH_3 from the T2 fragment was also observed. The molecular masses determined for these fragments (T2:988.5 and T3:541.5 Da) were in good agreement with the theoretical molecular masses (T2:988.4 and T3: 541.2 Da).

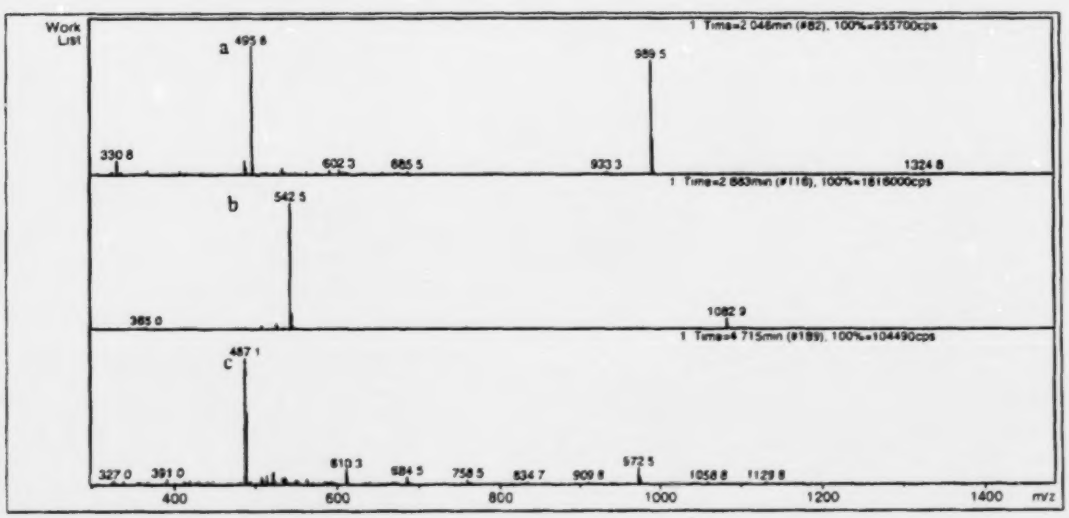

Figure 4. ESI-MS spectra obtained by France during the LC-MS analysis of the tryptic digestion of reduced MSA sample 001; a) T2 fragment, b) T3 fragment and c) T2-NH_3 fragment.

Sequencing of Tryptic Fragments

Sequence data on the enzymatic digestion products provides an additional level of confidence in the identification of the analyte. France carried out MS/MS experiments on two tryptic fragments (T2,T3). Figure 5 illustrates the ESI-MS/MS product spectrum acquired, by France, for the T3 fragment. Under conditions that promote the production of product ions, France acquired sequence information using b and y series ions from which the sequence of the T3 fragment (Asn-Tyr-Ser-Cys-NH_2) could be determined (Table II). Partial sequence information was also acquired for the T2 fragment.

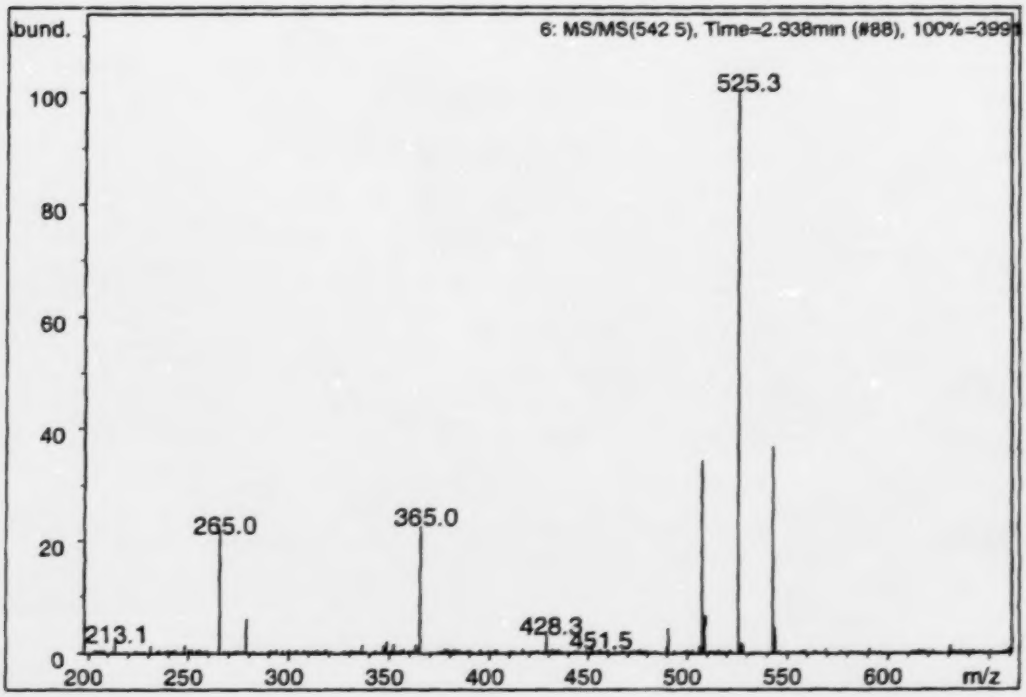

Figure 5. ESI-MS/MS product ion spectrum acquired by France for the $[M+H]^+$ precursor ion (542.5 Da) for tryptic fragment T3 of MSA Sample 001.

Table II. LC/MS/MS Results Obtained by France for T3 Fragment

b ions			y ions		
	Theoretical Mass (Da)	Observed Mass (Da)		Theoretical Mass (Da)	Observed Mass (Da)
b1	115.05				
b2	278.11	278.3	y3	428.14	428.3
b3	365.15	365.0	y2	265.07	265.0
b4	525.18	525.3	y1	178.04	

Additional Techniques

The participating laboratories used a variety of additional techniques to assist in the identification of MSA sample 001. Norway used amino acid analysis and determined that the amino acid composition was consistent with α-Conotoxin MI. Norway used N-terminal analysis to propose Gly-Arg-Cys-Cys-His-Pro-Ala- as the sequence starting at the N terminal. The United States used Edmans sequencing to propose the sequence Gly-Arg-Cys-Cys-His-Pro-Ala-Cys-Gly-Lys-Asn-Tyr-Ser-Cys-NH_2.

Germany and the United States each obtained reference samples of α-Conotoxin MI and compared the acquired mass spectrum with that acquired for MSA sample 001 and concluded that they were the same. The United States compared the molecular mass of sample and reductively alkylated sample to the molecular mass for α-Conotoxin MI and reductively alkylated α-Conotoxin MI and concluded they were the same.

Germany showed that the HPLC retention time for MSA sample 001 was the same as that for a reference sample of α-Conotoxin MI. Germany also spotted MSA sample 001 on a TLC plate that had been prepared with ninhydrin solution. The resulting reaction indicated that the sample was proteinaceous.

The Netherlands analyzed MSA sample 001 by proton NMR and TNDQCOSY NMR. The NMR data did not agree very well with literature data due to differences in the experimental setup. They felt there was partial agreement of the NMR spectra and literature data.

DRES MSA Sample 002

The second sample labelled "DRES MSA Sample 002" contained 1mg of apamin (Sigma, St. Louis, MO, USA). The amino acid sequence and molecular mass of apamin are given below.

Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH_2

Monoisotopic molecular mass:	2025.88661 Da
Average molecular mass:	2027.36779 Da
CAS number:	24345-16-2
Disulfide Bridges:	Cys 1-11 and Cys 3-15

Molecular Mass Determination

All the participating laboratories determined the molecular mass of the sample using electrospray ionization mass spectrometry. Figure 6 illustrates the electrospray mass spectrum acquired by Norway during the analysis of MSA sample 002. The mass spectrum contains $[M+2H]^{2+}$, $[M+3H]^{3+}$, and $[M+4H]^{4+}$ ions from which the molecular mass was determined. Table III lists the monoisotopic or average molecular mass obtained by each country. Once the laboratories obtained the molecular mass for the sample, and searched available databases, they were able to provisionally identify MSA sample 002 as apamin.

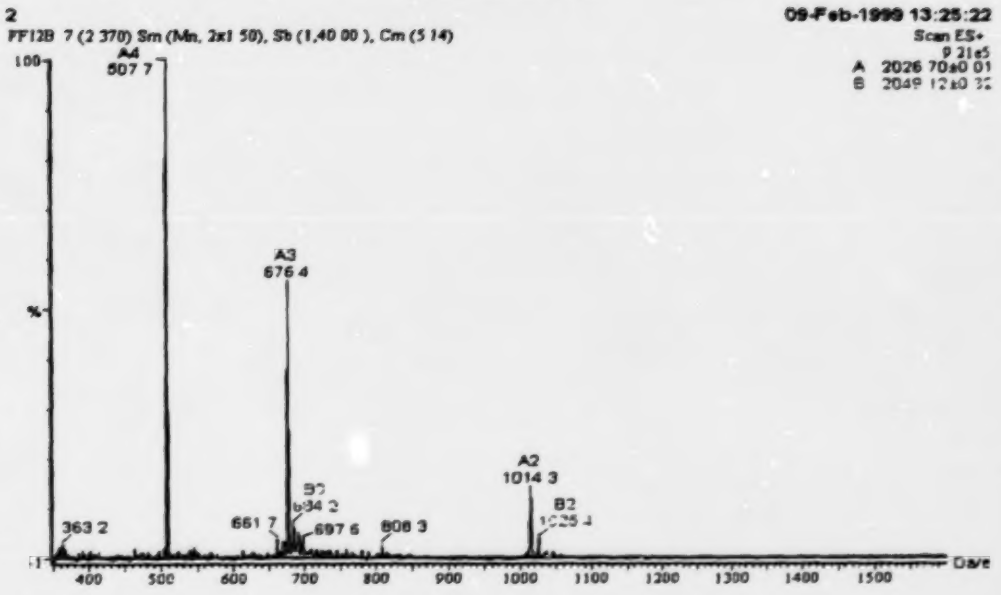

Figure 6. ESI-MS spectrum acquired by Norway during the analysis of MSA Sample 002.

Table III. Monoisotopic and Average Molecular Masses Determined for MSA Sample 002

Country	Instrument	Monoisotopic Molecular Mass 2025.88661 Da	Error ppm	Average Molecular Mass 2027.36779 Da	Error ppm
France	Bruker Ion Trap	2025.9	7		
Germany	HP 1100 MSD	2026	56		
Italy	PE Sciex	2026.0±0.1	56		
Netherlands	Micromass Q-TOF	2025.896±0.01	5		
Norway	Micromass Quattro			2026.70±0.01	330
U.S.	Finnigan TSQ7000			2027.53±0.6	80
Canada*	HP 1100 MSD	2026.0	56		

*host country

Reductive Alkylation

Once MSA sample 002 had been provisionally identified as apamin, a number of laboratories supported their identification by checking for the presence of disulfide bonds. Reduction of the disulfide bonds was in some cases followed by alkylation of the cysteine. The resulting change in the molecular mass of the analyte was determined by mass spectrometry. Figure 7 illustrates the ESI mass spectrum acquired by the United States during the analysis of MSA sample 002 following reduction with dithiothreitol. France, Germany, the Netherlands and the United States were able to confirm the presence of four cysteines and two intramolecular disulfide bridges.

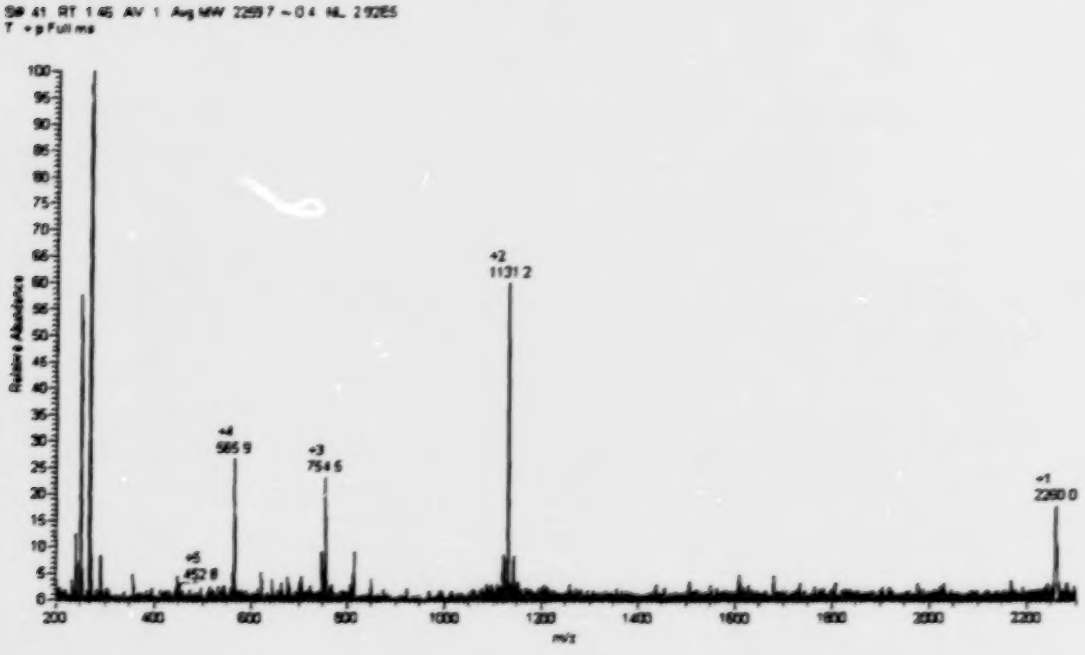

Figure 7. ESI-MS spectrum acquired by the United States during the analysis of MSA sample 002 following reduction and alkylation (iodoacetamide). Molecular mass was increased by 232 Da indicating the presence of two disulfide bridges.

Enzymatic Cleavage

France and the Netherlands both carried out tryptic digestion of MSA sample 002 and analyzed the digestion products. Figure 8 illustrates the total ion current chromatogram acquired by France during the analysis of the tryptic digestion of the reductively alkylated (iodoacetamide) MSA sample 002. Incomplete cleavage of the sample was observed and Table IV lists the mass map acquired for the digestion products. Figure 9 illustrates the ESI-MS spectra acquired for tryptic fragments T1, T4, T2 and T1+T2.

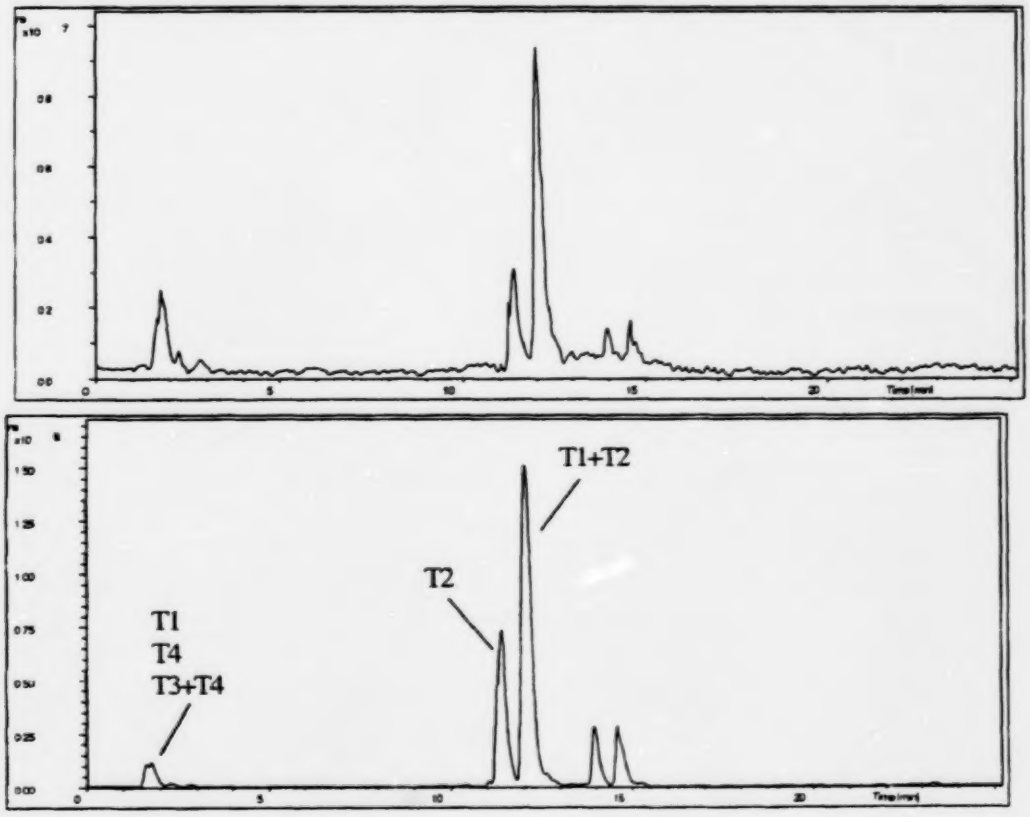

Figure 8. LC-ESI-MS; a) total ion current chromatogram and b) reconstructed ion chromatogram acquired by France during analysis of tryptic digestion products from reductively alkylated (iodoacetamide) MSA sample 002.

Table IV. Tryptic Digest Mass Map Obtained by France for MSA Sample 002

Retention Time (min)	Fragment Number	Fragment	Charge	Mol. Mass Observed	Mol. Mass Theoretical	Sequence
1.61	T4	15-18	+1	570.3	570.23	CQQH
1.61	T1	1-4	+1	580.3	580.21	CNCK
1.61	T3+T4	14-18	+1	726.5	726.33	RCQQH
11.39	T2	5-13	+1,+2	987.3	987.48	APETALCAR
11.97	T1+T2	1-13	+2,+3	1550.6	1549.68	CNCKAPETALCAR
13.92	T1+T2-NH3	1-13	+2	1533.4		
14.58	T1+T2-NH3	1-13	+2	1533.4		

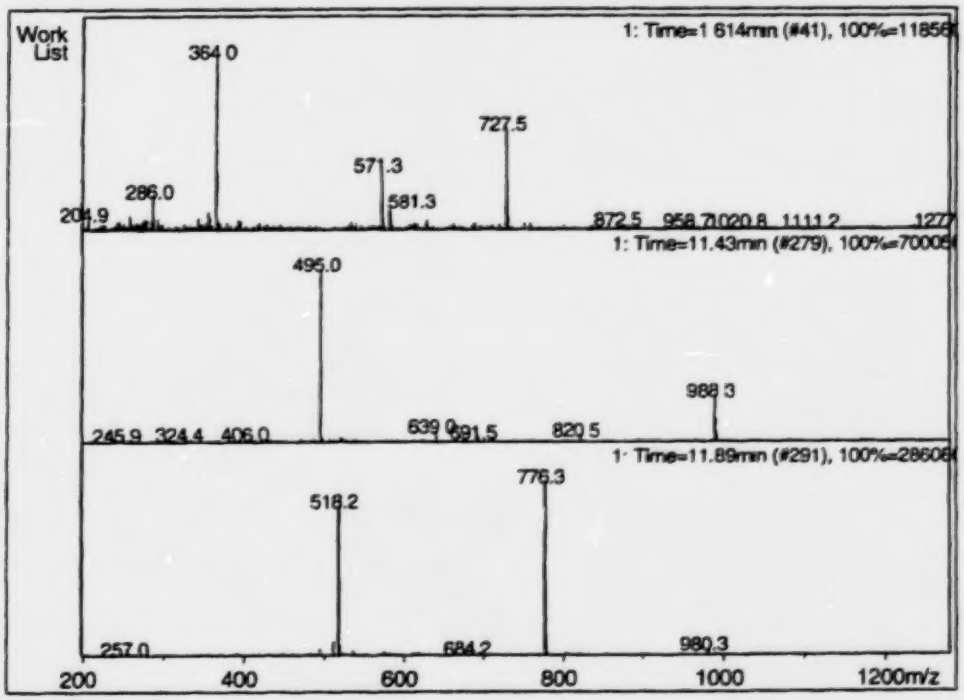

Figure 9. LC-ESI-MS spectra acquired by France for tryptic fragments; a) T1, T4, T3+T4, b) T2 and c) T1+T2 during analysis of reductively alkylated (iodoacetamide) MSA sample 002.

Sequencing of Tryptic Fragments

Sequence data on the enzymatic digestion products provides an additional level of evidence for confirming the identification of the analyte. France and the Netherlands carried out MS/MS experiments on the tryptic fragments from the digestion of MSA sample 002. Figure 10 illustrates the ESI-MS/MS product spectrum acquired, by the Netherlands, for the T2 fragment. Under conditions that promote the production of product ions, the Netherlands acquired sequence information using b and y series ions from which the sequence of the T2 fragment (Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg) could be determined. Both France and the Netherlands were able to acquire similar data from the other fragments.

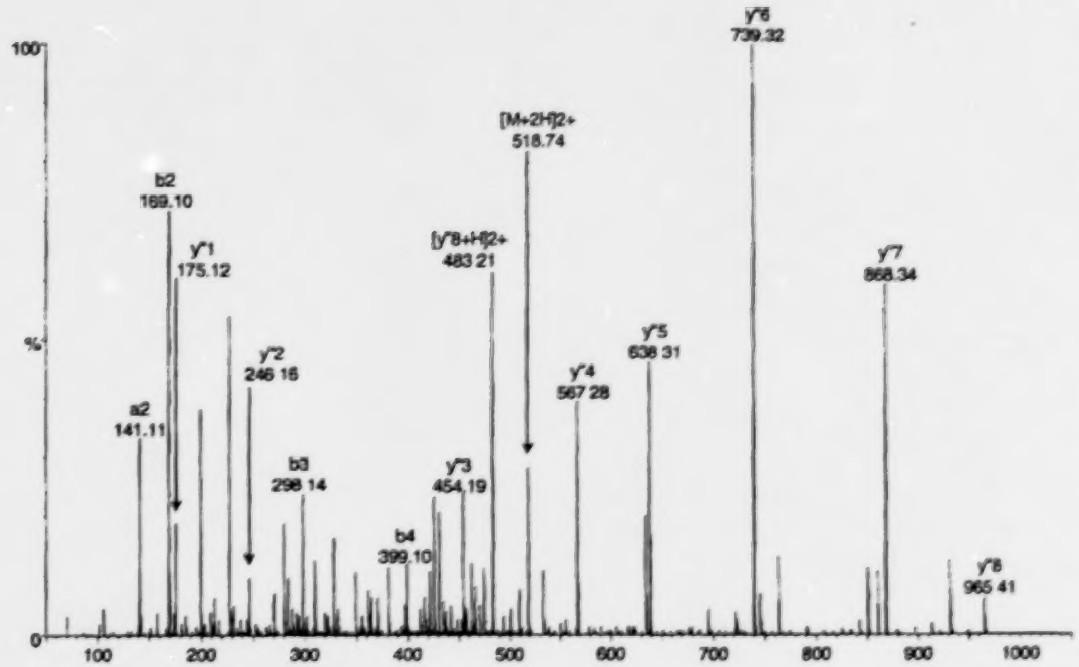

Figure 10. ESI-MS/MS product ion spectrum acquired by the Netherlands for the $[T2+2H]^{2+}$ precursor ion (518.74 Da) from reduced and alkylated (4-vinylpyridine) MSA sample 002.

Additional Techniques

The participating laboratories used a variety of additional techniques to assist in the identification of MSA sample 002. Norway used amino acid analysis and determined that the amino acid composition was consistent with apamin. Norway used N-terminal sequence analysis to propose Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu as the sequence starting at the N terminal. The United States used Edmans degradation to determine the sequence to be Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His.

The United States using mass spectrometry determined that a reference sample of apamin and MSA sample 002 had the same molecular weight. Germany acquired mass spectra for apamin and reduced apamin and compared them to the spectra acquired for MSA sample 002. They concluded that the spectra were the same.

The Netherlands compared the HPLC retention behaviour of apamin and MSA sample 002 under two mobile phase compositions. The retention times for the apamin and MSA sample 002 were the same using acetonitrile:water as the mobile phase under two different isocratic conditions. Germany demonstrated that apamin and MSA sample 002 had the same retention time using reverse phase liquid chromatography. Germany spotted MSA sample 002 on a TLC plate that had been prepared with ninhydrin solution. The resulting reaction indicated that the sample was proteinaceous.

Germany carried out initial molecular mass determinations using SDS-Page and gel permeation chromatography and determined that the molecular mass for MSA sample 002 was greater than 1350 Da.

The Netherlands compared the ^{1}H NMR data from an authentic reference sample of apamin and MSA sample 002. In addition, TNDQCOSY spectra were recorded in order to facilitate the assignment of resonances. The ^{1}H NMR data for the apamin and MSA sample 002 were identical.

DISCUSSION

Canada distributed two samples (MSA sample 001 and MSA sample 002) to the participating laboratories in January 1999. These samples had been selected from the SIBCA list of mid-spectrum agents and the laboratories were free to use any available technique in their identification of the samples. All the participating laboratories correctly identified MSA sample 001 as α-Conotoxin MI and MSA sample 002 as apamin. As part of their work on mid-spectrum agents, the SIBCA subgroup has developed an identification criteria for mid-spectrum agents. This criteria, found in Annex I, was used to evaluate the data reported by each of the laboratories. Table V lists the level of identification achieved by each laboratory.

Table V. Level of Identification Reached by Participating Laboratories

MSA Sample 001	Participating Laboratories						
	FR	GE	IT	NL	NO	US	CA[a]
Provisional Identification	✓	✓	✓	✓	✓	✓	✓
Confirmed Identification	✓[b]	✓		✓			✓[b]
Unambiguous Identification							
MSA Sample 002							
Provisional Identification	✓	✓	✓	✓	✓	✓	✓
Confirmed Identification	✓	✓		✓			✓
Unambiguous Identification				✓			

[a] Canada as the host country analyzed these samples prior to the training exercise as part of the sample selection process.
[b] only two tryptic fragments were observed.

All the laboratories were able to provisionally identify α-Conotoxin MI by determining the molecular mass by mass spectrometry and comparing the molecular mass to that of known mid-spectrum agent data (e.g. MSA database). Germany demonstrated that MSA sample 001 and an authentic reference sample of α-Conotoxin MI had the same chromatographic retention data, under two different experimental conditions. This along with determining molecular mass meant that Germany confirmed the identification of α-Conotoxin MI. France and the Netherlands were able to confirm the identification of α-Conotoxin MI by acquiring a mass map for the enzymatic (trypsin) digestion products of MSA sample 001.

All the laboratories were able to provisionally identify apamin by determining the molecular mass by mass spectrometry and comparing the molecular mass to that of known mid-spectrum agent data (e.g. MSA database). Germany and the Netherlands demonstrated that MSA sample 002 and an authentic reference sample of apamin had the same chromatographic retention data, under two different experimental conditions. This along with determining molecular mass meant that Germany had confirmed the identification of apamin. France and the Netherlands were able to confirm the identification of apamin by acquiring a mass map for the enzymatic (trypsin) digestion products of MSA sample 002. The Netherlands acquired the NMR spectrum of apamin and matched it to the spectrum for an authentic reference sample of apamin. This information, in addition to the chromatographic retention data and enzymatic mass map meant that the Netherlands had unambiguously identified apamin.

While electrospray ionization has expanded the range of compounds that can be analyzed by mass spectrometry, the mass spectra produced by this technique are not as reproducible as electron impact ionization, the traditional ionization method for CW agents. This training exercise allowed the comparison of ESI spectra obtained for the same sample by seven independent laboratories using different instruments and different experimental conditions. Tables VI and VII lists the instruments used by the laboratories and the intensity of the isotopic clusters observed. While the intensity of the ions varies considerably amongst the laboratories, the same ions are generally observed in each case.

Table VI. Intensity of Isotopic Clusters Obtained by Participating Laboratories for MSA Sample 001

Country	Instrument	Cone[b] (Volts)	$[M+H]^{+}$	$[M+2H]^{+2}$	$[M+3H]^{+3}$	$[M+4H]^{+4}$
France	Bruker Ion Trap	15	-	35	100	-
Germany	HP 1100 MSD	45	25	100	-	-
Italy	PE Sciex	70	1	60	100	40
Netherlands	Micromass Q-TOF	30-40	2	85	100	-
Norway	Micromass Quattro		1	100	40	-
U.S.	Finnigan TSQ7000		15	20	100	-
Canada[a]	VG AutoSpec	50	-	100	60	-

Table VII. Intensity of Isotopic Clusters Obtained by Participating Laboratories for MSA Sample 002

Country	Instrument	Cone[b] (Volts)	$[M+H]^{+}$	$[M+2H]^{+2}$	$[M+3H]^{+3}$	$[M+4H]^{+4}$
France	Bruker Ion Trap	15	-	1	20	100
Germany	HP 1100 MSD	45	15	100	25	-
Italy	PE Sciex	150	-	70	100	20
Netherlands	Micromass Q-TOF	30-40	-	25	80	100
Norway	Micromass Quattro		-	15	55	100
U.S.	Finnigan TSQ7000		15	10	20	100
Canada[a]	HP 1100 MSD	80	-	-	100	15

[a] host laboratory

[b] voltage applied in the ESI interface which influences analyte fragmentation. Name varies by instrument and may be called; sampling cone, fragmentor or skimmer.

The variation in the relative intensities of the isotopic clusters reported by the NATO laboratories suggests that at the current state of development of electrospray ionization it would be impractical to consider an ESI library based on matching ion intensities. While ESI libraries of mid-spectrum agents are required to assist in identification, a more promising solution appears to be a library based on simply the presence of ions indicative of the mass of the analyte.

CONCLUSIONS

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. All the participating laboratories were able to provisionally identify the first sample as α-Conotoxin MI and the second sample as apamin. The Netherlands and Germany through further experiments were able to confirm the identification of α-Conotoxin MI and apamin. In addition to the information required for confirmed identification, the Netherlands used nuclear magnetic resonance spectrometry and an authentic reference sample to unambiguously identify apamin.

The fact that the samples used during this training exercise were pure reference materials made sample preparation straightforward. The amount of sample being one milligram meant that, sensitivity was not an issue. Nonetheless this exercise was designed to determine if the NATO laboratories were able to identify unknown samples from the SIBCA list of mid-spectrum agents. All the particpating laboratories demonstrated the ability to correctly identify the two samples. In the future, training exercises could involve identification of mid-spectrum agents in trace amounts, in environmental or man-made matrices or focus on obtaining a higher level of identification.

Annex I

Identification Criteria for Mid-Spectrum Agents

Three levels of identification have been defined as follows to indicate the increasing level of certainty associated with each.

PROVISIONAL IDENTIFICATION: A mid-spectrum agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of known mid-spectrum agent data; or

II The molecular mass of the mid-spectrum agent, determined by MS, matches that of known mid-spectrum agent data; or

III A specific immunological assay registers a positive response.

CONFIRMED IDENTIFICATION: The identification of a mid-spectrum agent is confirmed when any two of the three criteria for provisional identification are met or:

I In the case of proteinaceous mid-spectrum agents, the molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that of known mid-spectrum agent data.

UNAMBIGUOUS IDENTIFICATION: Unambiguous identification provides the highest level of certainty required for the development of strategic and political positions. The identification of a mid-spectrum agent is unambiguous when the following conditions are met:

For non-proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match those for authentic reference standards acquired under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be carried out to confirm the molecular mass.

For proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of an authentic reference standard acquired under identical experimental conditions or a specific immunological assay registers a positive response; and

II The molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that for an authentic reference standard acquired under identical experimental conditions; and

III Sequence data for the mid-spectrum agent matches that for an authentic reference standard acquired under identical experimental conditions.